FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR
919.1002	09/856423

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/IL99/00541	**October 13, 1999**	**October 13, 1998**

TITLE OF INVENTION
HYDROCOLLOID COATING OF CELLS

APPLICANT(S) FOR DO/EO/US
Amos NUSSINOVITCH and Nir KAMPF

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☒ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Petition for Revival of an International Application for Patent Designating the U.S. Abandoned Unintentionally Under 37 C.F.R. 1.137(b)
Certificate of Express Mailing
Letter Re Priority

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/856423	PCT/IL99/00541	919.1002

24. The following fees are submitted:.		CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :			
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1000.00**		
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	**$860.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$710.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$690.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	**$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =		**$860.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	20 - 20 =	0	x $18.00	**$0.00**	
Independent claims	3 - 3 =	0	x $80.00	**$0.00**	
Multiple Dependent Claims **(check if applicable).**			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$860.00**	
☒ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$430.00**	
SUBTOTAL =				**$430.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$430.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$430.00**	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$430.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-0513** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

STEINBERG & RASKIN, P.C.
1140 Avenue of the Americas
15th Floor
New York, New York 10036-5803

SIGNATURE

Martin G. Raskin
NAME

25,642
REGISTRATION NUMBER

May 21, 2001
DATE

919.1002

UNITED STATES PATENT AND TRADEMARK OFFICE

Re:	Application of:	Amos NUSSINOVITCH, et al.
	Serial No.:	Not yet known
	Filed:	Simultaneously
	For:	**HYDROCOLLOID COATING OF CELLS**

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

May 21, 2001

Sir:

Prior to examination and calculation of the filing fee, please amend the above-identified application as follows.

IN THE CLAIMS:

Please amend the claims as follows.

1. (Amended) A method of coating a cell [characterised in that] comprising the steps of:

placing the cell [is placed] in a solution of hydrocolloid [and, after];

removing the cell from the solution of hydrocolloid [solution, is placed]; and

placing the cell in a cross-linking solution after removing the cell from the solution of hydrocolloid, [to] thereby [provide] providing the cell with a thin coating of the hydrocolloid.

3. (Amended) A method as defined in Claim 1, wherein the [alginate] hydrocolloid is Na-alginate.

4. (Amended) A method as defined in Claim 1, wherein the hyrocolloid is [LMP] low-methoxy pectin (LMP).

5. (Amended) A method as defined in Claim 1, wherein the hydrocolloid is [selected among] κ-carrageenan or ι-carrageenan.

6. (Amended) A method as defined [in any of Claims 1 to 5, characterised in that] Claim 1, wherein the hydrocolloid solution is in [CAMMR] Calcium Adjusted Modified Marc's Ringer (CAMMR) solution.

7. (Amended) A method as defined [in any of Claims 1 to 6] in Claim 1, wherein the cell is a [Xenopus laevis] Xenopus laevis egg and embryos.

8. (Amended) A method as defined in [any of Claims 1 to 7] Claim 1, wherein the cross-linking solution is a solution of Ca, Ba or K ions.

11. A method as defined in [any of Claims 1 to 10] Claim 1, wherein said thin layer coating of hydrocolloid is up to about 50 μm in thickness.

12. (Amended) A method of postponing hatching of [Xenopus laevis] Xenopus laevis embryos comprising the steps of:

applying a thin coating of [an] a hydrocolloid to a [Xenopus laevis] Xenopus laevis egg; and

cross-linking said hydrocolloid.

13. (Amended) A method as defined in [any of Claims 1 to 3 and 6 to 12] Claim 1, wherein the alginate has a high [M] mannuronic acid (M) content.

14. (Amended) A method as defined in Claim 13 wherein the [M] mannuronic acid (M) content of the alginate is from about 29 to about 61 %.

15. (Amended) A cell having a thin coating of a hydrocolloid [according to any of the Claims 1 to 14].

Please add the following new claims.

16. (New) A method as defined in Claim 12, wherein the hydrocolloid is an alginate.

17. (New) A method as defined in Claim 12 wherein the alginate has a high mannuronic acid (M) content.

18. (New) A cell according to Claim 15, wherein the hydrocolloid is alginate, Na-alginate, low-methoxy pectin (LMP), κ-carrageenan or ι-carrageenan.

19. (New) A cell according to Claim 15, wherein the cell is a Xenopus laevis egg and embryos.

20. (New) A cell according to Claim 18, wherein the alginate has a mannuronic acid (M) content of from about 29 to about 61 %.

Attached hereto please find a "Marked-Up version of the Claims as Amended" including the amendments made to claims 1, 3-8 and 11-15 above as well as new claims 16-20 added above.

In addition, also attached hereto, please find an "Un-Marked Version of the Claims as Amended" including the amendments made to claims 1, 3-8 and 11-15 above as well as new claims 16-20 added above. Please note, additions to the claims are denoted by underlining and deletions from the claims are denoted by bracketing.

REMARKS

It is respectfully requested that the replacement of substitute sheets of Figures made pursuant to Rule 26 under Article 34 in response to the International Preliminary Examination Report be entered for purposes of the present application.

Claims 1, 3-8 and 11-15 have been amended to correct minor inconsistencies, to more clearly define the invention and to remove multiple dependencies in order to reduce the filing fee. New claims 16-20 have been added which are commensurate in scope with original claims 1-15 and which are fully supported by the written description.

An early and favorable action on the merits is earnestly solicited.

Respectfully submitted,

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Encls.
- Marked-Up Version of the Claims as Amended
- Un-Marked Version of the Claims as Amended

Un-Marked Version of the Claims as Amended 919.1002

1. (Amended) A method of coating a cell comprising the steps of:

placing the cell in a solution of hydrocolloid;

removing the cell from the solution of hydrocolloid; and

placing the cell in a cross-linking solution after removing the cell from the solution of hydrocolloid, thereby providing the cell with a thin coating of the hydrocolloid.

3. (Amended) A method as defined in Claim 1, wherein the hydrocolloid is Na-alginate.

4. (Amended) A method as defined in Claim 1, wherein the hyrocolloid is low-methoxy pectin (LMP).

5. (Amended) A method as defined in Claim 1, wherein the hydrocolloid is κ-carrageenan or ι-carrageenan.

6. (Amended) A method as defined Claim 1, wherein the hydrocolloid solution is in Calcium Adjusted Modified Marc's Ringer (CAMMR) solution.

7. (Amended) A method as defined in Claim 1, wherein the cell is a Xenopus laevis egg and embryos.

8. (Amended) A method as defined in Claim 1, wherein the cross-linking solution is a solution of Ca, Ba or K ions.

11. A method as defined in Claim 1, wherein said thin layer coating of hydrocolloid is up to about 50 μm in thickness.

12. (Amended) A method of postponing hatching of Xenopus laevis embryos comprising the steps of:

applying a thin coating of a hydrocolloid to a Xenopus laevis egg; and

cross-linking said hydrocolloid.

13. (Amended) A method as defined in Claim 1, wherein the alginate has a high mannuronic acid (M) content.

14. (Amended) A method as defined in Claim 13 wherein the mannuronic acid (M) content of the alginate is from about 29 to about 61 %.

15. (Amended) A cell having a thin coating of a hydrocolloid.

16. (New) A method as defined in Claim 12, wherein the hydrocolloid is an alginate.

17. (New) A method as defined in Claim 12 wherein the alginate has a high

mannuronic acid (M) content.

18. (New) A cell according to Claim 15, wherein the hydrocolloid is alginate, Na-alginate, low-methoxy pectin (LMP), κ-carrageenan or ι-carrageenan.

19. (New) A cell according to Claim 15, wherein the cell is a Xenopus laevis egg and embryos.

20. (New) A cell according to Claim 18, wherein the alginate has a mannuronic acid (M) content of from about 29 to about 61 %.

1. (Amended) A method of coating a cell [characterised in that] comprising the steps of:

placing the cell [is placed] in a solution of hydrocolloid [and, after];

removing the cell from the solution of hydrocolloid [solution, is placed]; and

placing the cell in a cross-linking solution after removing the cell from the solution of hydrocolloid, [to] thereby [provide] providing the cell with a thin coating of the hydrocolloid.

3. (Amended) A method as defined in Claim 1, wherein the [alginate] hydrocolloid is Na-alginate.

4. (Amended) A method as defined in Claim 1, wherein the hyrocolloid is [LMP] low-methoxy pectin (LMP).

5. (Amended) A method as defined in Claim 1, wherein the hydrocolloid is [selected among] κ-carrageenan or ι-carrageenan.

6. (Amended) A method as defined [in any of Claims 1 to 5, characterised in that] Claim 1, wherein the hydrocolloid solution is in [CAMMR] Calcium Adjusted Modified Marc's Ringer (CAMMR) solution.

7. (Amended) A method as defined [in any of Claims 1 to 6] in Claim 1, wherein

the cell is a [Xenopus laevis] Xenopus laevis egg and embryos.

8. (Amended) A method as defined in [any of Claims 1 to 7] Claim 1, wherein the cross-linking solution is a solution of Ca, Ba or K ions.

11. A method as defined in [any of Claims 1 to 10] Claim 1, wherein said thin layer coating of hydrocolloid is up to about 50 μm in thickness.

12. (Amended) A method of postponing hatching of [Xenopus laevis] Xenopus laevis embryos comprising the steps of:

applying a thin coating of [an] a hydrocolloid to a [Xenopus laevis] Xenopus laevis egg; and

cross-linking said hydrocolloid.

13. (Amended) A method as defined in [any of Claims 1 to 3 and 6 to 12] Claim 1, wherein the alginate has a high [M] mannuronic acid (M) content.

14. (Amended) A method as defined in Claim 13 wherein the [M] mannuronic acid (M) content of the alginate is from about 29 to about 61 %.

15. (Amended) A cell having a thin coating of a hydrocolloid [according to any of the Claims 1 to 14].

16. (New) A method as defined in Claim 12, wherein the hydrocolloid is an alginate.

17. (New) A method as defined in Claim 12 wherein the alginate has a high mannuronic acid (M) content.

18. (New) A cell according to Claim 15, wherein the hydrocolloid is alginate, Na-alginate, low-methoxy pectin (LMP), κ-carrageenan or ι-carrageenan.

19. (New) A cell according to Claim 15, wherein the cell is a Xenopus laevis egg and embryos.

20. (New) A cell according to Claim 18, wherein the alginate has a mannuronic acid (M) content of from about 29 to about 61 %.

HYDROCOLLOID COATING OF CELLS

Technical Field

This invention relates to the coating of cells, and more particularly, this invention relates to the application of a thin hydrocolloid-based film on individual cells.

Background Art

It is known that cells can be entrapped within a gel matrix. A wide range of characteristics are attributed to gels as an entrapment medium. On the one hand, they include macromolecules held together by relatively weak intermolecular forces, such as hydrogen-bonding or ionic cross-bonding by divalent or multivalent cations. On the other hand, strong covalent bonding, where the lattice in which the cells are entrapped is considered as one vast macromolecule, is limited only by the particle size in the immobilized cell preparation [55, 48]. The major categories of entrapment have been reviewed (22, 21, 45, 48, 50]. They include some commonly used, single-step entrapment methods, such as the simple gelation of macromolecules by lowering or raising temperatures using hydrocolloids such as agar [20], agarose [59], -carrageenan [23, 20, 27, 57, 35], chitosan [56, 41, 54, 27), gelatin and egg whites [27], among others. These preparations regularly suffer from low mechanical strength and possible heat damage. Another simple single-step entrapment method is the ionotropic gelation of macromolecules by di- and multivalent cations, using alginate [30, 25, 27, 42, 24, 38] and Low -Methoxy-Pectin (LMP), among others. The limitations of such systems are low mechanical strength and breakdown in the presence of chelating agents.

During immobilization, 10^4 to 10^9 microorganisms (bacteria, yeast or fungal spores having a maximal diameter of 5 microns) can be entrapped within 1 ml of gelling agent [48,49]. In such cases, a maximal 6.5% of the volume is occupied by the microorganisms. In other words, 93.5% of the volume is not occupied by the cells, or, if the cells are evenly distributed throughout the gel volume, then each individual cell is coated by a very thick layer of gel in comparison to its own natural dimensions.

Disclosure of Invention

In contrast, coating of a single cell with a thin layer of film, comprising only a fraction of its diameter has advantages. The difference between coating and entrapping is the thickness of the coating layer being very thin in the former, thick in the latter. Taking this definition into

account, it seems that most, if not all reports on "coating" are in fact describing cell entrapment within a gel matrix [43, 51, 31]. To obtain a true coating, special micro-coating procedures must be used. By this invention, we have developed such a procedure. For purposes of demonstrating this procedure, we chose one of the biggest cells in nature: the fertilized frog egg.

We were able to study the influence of different hydrocolloid coatings (thin films that are glued to the outer surface of the egg) on the survival of Xenopus laevis embryos, in terms of their biological advantages and disadvantage under different conditions of cross-linking and storage. The hydrocolloid coating of the embryos: (a) postponed hatching and extended survival rates, (b) protected the embryos from microbial contamination, (c) protected the embryos from hazardous material produced or introduced into the media, and (d) acted as an inhibitor against damage during freezing and thawing.

Brief Description of Drawings

Fig. 1 is a graph showing the effect on survival after hatching of X. laevis embryos vs. elapsed time by alginate type (the ± 5% bar indicates the experimental uncertainty);

Fig. 2 is a graph showing the effect on survival after hatching of X. laevis embryos vs. elapsed time in the case of storage condition # 1 by type of cross-linking agent (stippled areas emphasize coating with which no significant difference between survival was detected);

Fig. 3 is a graph showing the influence of salt type and concentration on the thickness of the alginate coating and the embryo's jelly coat 4 hours after fertilization;

Fig. 4 is a SEM micrograph of X. laevis embryo; 1) alginate coating, 2) jelly coat, 3) embryo; and

Fig. 5 is a graph showing the effect on survival after hatching of X. laevis embryos vs. elapsed time in the case of storage condition # 2 by type of cross-linking agent (stippled areas emphasize coating with which no significant difference between survival was detected).

Fig. 6 is a graph showing the effect of hydrocolloid coatings on the survival of X. laevis embryos vs. elapsed time. a, b, c and d represents the significant statistical difference.

Fig. 7 demonstrates the effect of hydrocolloid coating on embryo Jelly Coat (JC) thickness vs. time.

Fig. 8 demonstrates the influence of hydrocolloid coating thickness on the survival of X. laevis embryos.

Fig. 9 is a SEM micrograph of X. laevis coated embryos in cross section: (a) LMP, (b) κ-carrageenan, (c) alginate, (d) ι-carrageenan. 1) Hydrocolloid coating. 2) Jelly coat. 3) Embryo.

Fig. 10 is a SEM micrograph of coated and noncoated X. laevis embryos: (a) LMP, (b) alginate, (c) ι-carrageenan, (d) κ-carrageenan, (e) control.

Best Mode for Carrying Out the Invention

Frog maintenance, egg ovulation and fertilization procedure was carried out according to M. Wu and J. Gerhart, (1991), Hedrick and Nishihara, (1991), and Nieuwkoop and Farber, 1994. Specifically, sexually mature Xenopus laevis (South African clawed toads) were maintained in the laboratory under constantly controlled conditions. Room and water temperatures were maintained at 18±1°C using an air conditioner. Animals were exposed to a 12/12 h light/dark period, to keep oocytes at a mature stage. Animals were fed with chick liver or heart twice a week, and water was changed after feeding with aged tap water [1].

Females were intramuscularly injected with 1000 IU of human chorionic gonadotropin (hCG) (N.V. Organon Oss, Holland). Egg-laying began ~18 h after injection. When signs of laying were observed, some of the eggs were squeeze-stripped into a petri dish and immediately fertilized.

Fertilized eggs were obtained as follows: fresh testes were dissected from an *X. laevis* male and kept in full-strength Modified Marc's Ringer (MMR) solution (full-strength MMR = 100 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, adjusted to pH 7.4). Testes were then mixed with ovulated eggs for 10 sec and one-third-strength MMR solution was added. Fertilized and non-fertilized eggs were separated by visual inspection and only eggs showing the first cleavage (~1.5 h after fertilization) were chosen for further manipulation. Embryo developmental stages were monitored under a binocular lens and compared to the Normal Table of X. laevis (Daudin) [3].

After fertilization, the embryos were dropped into a 1% alginate solutginate solution made b Na-Alginate in one-third-strength Calcium Adjusted MMR (CAMMR)solution (same concentration as 1/3 MMR except of reduced calcium content to 0.22 mM to eliminate accidental cross-linking reaction). Alginate compositions, supplied by the manufacturer, are given in Table 1. Other hydrocolloids used for coating were 1% low-methoxy pectin (LMP),

1% κ-carrageenan or 1% ι-carrageenan dissolved in CAMMR solution. Embryos were then sucked into a 1.5-mm diameter tube and dropped into the cross-linking agent. The alginates were cross-linked with either Ca or Ba ions (available as $CaCl_2$ or $BaCl_2$ salts (Sigma Chemical Co., St. Louis, MO)) at three different concentrations: 0.25, 0.5 or 1% (w/w) (equal to 25, 50 and 100 mM $CaCl_2$, respectively or 12.5, 25 and 50 mM $BaCl_2$, respectively). LMP and ι-carrageenan were cross-linked with 0.5% Ca (available as $CaCl_2$ salt; Sigma Chemical Co., St. Louis, MO) equal to 50 mM $CaCl_2$. κ-Carrageenan was cross-linked with 0.5% K (available as KCl salt; Sigma Chemical Co., St. Louis, MO) equal to 67 mM KCl. The salts were dissolved in one-third-strength CAMMR solution to maintain the egg's physiological osmotic pressure. After dipping in the cross-linking agent for 20 seconds, coated embryos were washed once and then stored in sterile one-third-strength CAMMR solution.

Embryos coated with alginate were kept for 196 hours under one of three different storage conditions:

1) Closed (sterile) petri dishes containing 30 embryos in 50 ml of one-third-strength CAMMR solution at a volume ratio of 1.6 ml per embryo.

2) Open petri dishes containing 30 embryos in 50 ml of one-third-strength CAMMR solution at a volume ratio of 1.6 ml per embryo.

3) Aerated, circulated, stirred and dechlorinated tap water at a volume ratio of 85 ml per embryo.

All experiments were conducted in triplicate at 20 ±1C (maintained by air conditioner), and the embryo's developmental stages were monitored after fertilization. Every 4 to 8 hours, larval hatching from the natural jelly coat or artificial alginate coating was determined. Survival of the larvae was determined by observing movement. Dead or non-hatching embryos were not included in the survival calculations. Percent survival after hatching was calculated as the surviving hatched larvae out of the total number of embryos.

Changes in the egg's natural jelly coat's dimensions and the artificial alginate coat's thickness were measured up to -48 hours under a binocular using a grid-measuring lens. Scanning electron microscopy of eggs was performed in a Jeol JSM 35C (Tokyo, Japan). Immediately after laying, the egg was glued to a polypropylene stub and tested under low-vacuum conditions.

Changes in the egg's natural JC dimensions or the artificial hydrocolloid coat's thickness were measured up to ~48 h under a binocular microscope using a grid-measuring lens.

Determination of hydrocolloid mechanical properties: Preparation of hydrocolloid-gel films: 0.5% (w/w) Na-alginate, 1% (w/w) LMP, 1 % (w/w) ι-carrageenan and 1% (w/w) κ-carrageenan powders were dissolved in one-third-strength CAMMR solution. A cellulose-acetate sleeve was filled with 2 ml hydrocolloid solution to form an ~1-mm thick layer. Gelation of the alginate, LMP and ι-carrageenan occurred after dipping the sleeve in a 0.5% CaCl2 solution bath. Gelation of the κ-carrageenan occurred after dipping the sleeve in a 0.5% KCl solution bath. Mechanical tests are conducted after 24 h of storage at 24°C.

Mechanical tests: Gel height and width were determined by caliper (Mitutoyo, Tokyo, Japan). Gel thickness was determined by micrometer (Mitutoyo, Tokyo, Japan). The tip of the specimen was mounted on an Instron UTM, model 1100 (Instron Corp., MA) and set in tension mode. All specimens were deformed at a constant deformation rate of 10 mm/min. Data-gathering and processing were performed with a 486 IBM-compatible computer interfaced with the UTM. The force-deformation curves of the specimens were transformed into corrected ("true") stress, σc, and Hencky's ("true") strain, εH, by the following transformations:

$$\sigma c = F(Lo+\Delta L)/(AoLo)$$

where F is the force, Ao and Lo the initial cross-sectional area and length of the specimen, respectively, and ΔL the absolute deformation, and

$$\varepsilon H = \ln((Lo+\Delta L)/Lo)$$

The deformability modulus, ED, was calculated from the linear portion of the stress-strain curves.

Statistics: Results of survival after hatch, JC and hydrocolloid coating thicknesses, hydrocolloid mechanical properties and tension of the hydrocolloid solutions were statistically tested by ANOVA (JMP software, SAS Institute Inc.)

Microbial mass in the embryo's one-third-strength CAMMR medium was assayed as presence of microbial ATP using a dairy products sterility test kit (LUMAC® B.V. Landgraaf, The Netherlands). Free ATP was degraded by adding 10 l of ATPase enzyme (SOMASE™) to 50 l of embryo medium and incubating at room temperature for 15 min. Then, the enhancement of microbial cell wall and membrane permeability to ATP was established by adding L-NRB® reagent for 30 seconds. Finally, the presence of microbial ATP was assayed for 10 seconds by coupled reaction of luciferin-luciferase enzymes [4]. Emitted light was measured by luminescence photometer (BIOCOUNTER®,m 2500, Landgraaf, The Netherlands). The correlation between the actual number of microorganisms and the light emitted from the above-mentioned assay was found using a total plate count culture composed of 1% agar (Difco, MI, USA), 0.5% yeast extract (Difco) and 3% tryptic soy broth (Difco).

Biological oxygen demand (BOD) was measured every 24 hours during the 196-hour experiments. An oxygen-temperature electrode was used for BOD detection and was connected to a portable printing and logging dissolved-oxygen meter model HI 9141 (Hanna Instruments, Woonsocket, RI, USA). Oxygen levels in the embryo medium (± 0.01 ppm) were recorded at the specified times.

Changes in the pH values of the embryo storage medium were detected using a pH meter model HI 9141 (Hanna Instruments, Woonsocket, RI, USA).

Mineral content was determined in the alginate-jelly coat (removed manually from the embryos) and within the embryos over time, elapsed from fertilization and coating. Each sample was prepared from five embryos and kept in

a microfuge tube at -20°C until analysis. Preparations of 1/3 CAMMR, dechlorinated tap water, cross linking agents were also analyzed.

The contents of each microfuge tube were defrosted, dissolved in concentrated nitric acid and transferred to graduated, 50-ml polypropylene vessels. The microfuge tubes were further rinsed with a fresh portion of acid, adding a total volume of 1 ml to each sample. Two blanks were processed in parallel. The vessels were fitted with screw caps and transferred to a temperature-controlled microwave oven. Samples were subjected to three digestion cycles of 20 min each, at 450 W and 95°C. The vessels were allowed to cool for 10 min between cycles, and at the conclusion of the digestion program were brought to room temperature and uncapped. The volume was brought to 10 ml with deionized water.

Analysis was conducted on portions of these solutions, versus multielement standards, prepared using the same matrix. All elements were determined in the tested solutions by inductively

coupled plasma atomic emission spectrometry (ICP-AES), using a model "Spectroflame Modula E" ICP-AES from Spectro (Kleve, Germany), with a standard cross-flow nebulizer and a fixed End-On-Plasma torch. The power level was 1.2 kW, with a coolant flow of 15 l/min, an auxiliary flow of 0.5 l/min and a nebulizer flow of 0.5 l/min.

In a first set of experiments, X. laevis fertilized eggs were coated with three different types of alginate. The properties of these alginates are summarized in Table 1: they differed with respect to their molecular weights, viscosities, gel strengths and the content ratios of guluronic (G) to mannuronic (M) acid. The molecular weight, and the proportion and arrangement of M and G are expected to affect a particular algimate's behavior. The percentage of M in the alginates used for coating ranged from 29 to 35 in the alginates extracted from *Laminaria hyperborea*, to 61 in the alginate extracted from *Macrocystic pyrifera.* Each egg was covered with a thin layer of calcium- or barium- alginate gel. Alginate was chosen for this study because its coatings are easy to produce as discussed above, and they have been used successfully for many products [5-9]. Moreover, as can be assumed from the vast experience accumulated from cell-entrapment experiments, alginate gels maintain cell viability [10].

Company	Product Name	Origin	Molecular Weight	Viscosity	Gel Strength	% Dry Solids	G:M Ratio
Sigma Chemical Co., St. Louis, USA	Alginic Acid Sodium salt, Low visc.	Macrocystic Pyrifera	60,000-70,000	22% (cP) at a conc. of 2%	Not detected	88	39:61
Pronova Biopolymer . a.s. Drommer, Norway	Alginic Acid Sodium salt (Protanal LF 10/60)	Laminaria Hyperborea	123000	50 (cP) at a conc. of 1%	59.9 g (water)	87.8	71:29
Pronova Biopolymer . a.s. Drommer, Norway	Alginic Acid Sodium salt (Protanal LF 20/60)	Laminaria Hyperborea	185000	126 (cP) at a conc. of 1%`	56.9 g (water)	86.5	65:35

Table 1: Deferent Alginate compositions (supplied by the manufacturer)

The properties of others of the hydrocolloids are summarized in Table 2. They differed in their chemical structure and composition, in the way they produced gels, in the cross-linking agents used for gelation, and in the properties of the films they produced.

Product Name	Source	Molecular Weight (Dalton)	Viscosity (cP)	Composition	Company
Alginic acid sodium salt, low visc.	Macrocystis pyrifera	60,000-70,000	228 at a concentration of 2%	39% glucoronic acid and 61% mannuronic acid	Sigma Chemical Co. St. Louis, USA
ι-Carrageenan	Eucheuma spinosa	250,000	288 at a concentration of 1.5%	32% ester sulfate and 30% 3,6 anhydride-galactose	Sigma Chemical Co. St. Louis, USA
κ-Carrageenan	Eucheuma cottonii	154,000	23 at a concentration of 1.5%	25% ester sulfate and 34% 3,6 anhydride-galactose	Sigma Chemical Co. St. Louis, USA
GENU pectin type LM-5 CS	Citrus peel	80,000-100,000	20 at a concentration of 1%	Methyl ester (<10%) of polygalacturonic acid	Hercules Incorporated Lille Skensved, Denmark

Table 2: The properties of low-methoxy pectin, alginate, ι and κ-carrageenan hydrocolloids (supplied by the manufacturers).

Alginate was chosen for this study because its coatings are easy to produce and they have been used successfully for many products (Nussinovitch and Kampf, 1993; 9, 6, Kampf et al., 1998). Moreover, as can be assumed from the vast experience accumulated from cell-entrapment experiments, alginate gels maintain cell viability [10, 43]. LMP is similar to alginate in its cross-linking mechanism, making a comparison between the two of interest. The use of LMP

for coatings is also not new such coatings were being used for nuts and dried dates almost 50 years ago (Swenson et al., 1953). Carrageenans were included in this research for their different gelation mechanisms and the possibility of achieving coatings with favorable properties. Carrageenan-based coatings were developed by Mitsubishi International Corp. (IFT, 1991) but aside from their designation for fresh produce, no information on them is available (Baldwin, 1994). Other carrageenan coatings have been used to retard moisture loss from coated foods, including cheeses (Torres et al., 1985, 9).

We performed our first coating and storage experiments under so-called "harsh" conditions (storage conditions # 1), thereby making it easy to conclude whether a particular coating is beneficial, relative to uncoated embryos: the conditions were modified from those recommended by Wu and Gerhart,(1991), and Phillips,(1979). However, we increased the proportion of eggs to medium solution such that instead of including 10 embryos per 50 ml medium, we introduced 30 embryos per 50 ml and allowed only passive natural aeration to take place, thereby increasing the stress on the coated embryos. Embryo's medium was contained within sterile container and conditions. Coated embryos were also introduced into the same medium, except that the sterile medium was exposed to non-sterile conditions (storage conditions # 2). Coated embryos were also maintained under the "ideal" conditions reported by

Wu and Gerhart (1991) and Phillips (1979) to check their performance in a more favorable environment (storage conditions # 3).

The survival of embryos vs. time under storage conditions #1 is shown in Fig. 1. The survival percentage is equivalent to the accumulated number of hatching embryos to a maximal or asymptotic survival value, and is the number of embryos left after they begin to die. The accumulated survival percentage [1] of non-coated embryos was 4.6, 54 hours after fertilization, increasing to 66 after 60 hours (Fig. 1). Percent survival then decreased to 41 after 78 hours and reached an asymptotic value of 30 between 84 and 196 hours. Reduced survival percentages could be due to the secretion of nitrates or other substances into the medium by the developing embryos. In parallel to the survival-prospects study, embryo development was monitored by comparing their developmental stage (observed through a binocular lens) to that of non-coated embryos [3]. No difference between the two was observed, implying that the coating film does not hamper embryo development.

A large difference between the alginates was observed: the alginate with a high proportion of M held better prospects for embryos hatching. The asymptotic survival value for the high-M coating was 53-56% vs. 22 to 32% for the high-G coatings. This is due to the fact that the higher the G content, the stronger the gel (i.e. the film coating the embryo). In other words, a high G content and long G blocks confer high calcium reactivity and the strongest gel-forming potential to the alginates. Coated embryos appeared to develop in a normal fashion, similar to non-coated embryos, however the strong coating (high G) prevented hatching embryos from bursting the thin coating film and thus 120 hours after fertilization, they perished. No significant differences were found between the two alginates extracted from the *L. hyperborea.* Significant differences in survival rate were observed between the high-M and high-G alginates. The hatching process in X. laevis embryos toad consists of two temporally distinct phases [12]. Phase 1 appears to be a physical process, which ruptures jelly-coat layers J3 and J2. This exposes J1 to the outside medium, in which is partial soluble, and permitting its gradual dissolution. Phase 2 is a result of both physical and chemical (proteolytic enzyme secretion) processes. Mobility helps the embryo emerge from its jelly coat, but is not enough to break through a high-G coating film.

An additional difference was observed between the uncoated and coated embryos. The former reached their maximal survival rate a short time after hatching began. For the coated systems, a maximal value was reached 25 hours later. This means that some delay in hatching was effected by the coating process. This delay is important for longer-term experiments with embryos. Another advantage is that the embryo hatches at a much more developed stage relative to non-coated embryos. Thus the embryo is less prone to mechanical damage or microbial contamination. Bacteria have been reported to stick to the surface of the J3 outer layer of the jelly coat and that removal greatly reduces their number. Coating embryos could therefore eliminate the need for including neomycin sulfate in the media [12].

In addition one of the roles of the natural Jelly coat in amphibians is to serve as a heat accumulator, especially in high attitude location where the fertilized eggs are exposed to lower temperatures [18]. Coating the embryo with an artificial gel layer would decrease heat loss by insulating the embryo from its surrounding. Moreover, the artificial gel coating could condense the light rays as they heat the embryo. As stated by Beattie (1980), larger gelatinous capsules around the eggs may increase their chances of survival.

Based on these preliminary coating experiments and the conclusion that embryos are not capable of breaking through films with a high G content, further coating experiments were carried out only with the high-M alginate.

Sodium alginate can be cross-linked with several divalent ions. We checked the performance of the high-M alginate coating after cross-linking with different concentrations of Ca or Ba. The embryos were immersed in the same medium (one-third CAMMR solution) but the conditions were not sterile, and the embryos were prone to microbial contamination. Fig. 2 demonstrates the relative successes of the different coatings.

Coatings produced with alginate cross-linked with 0.25 and 0.5% CaCl2 were most successful, i.e. a higher percentage of hatching and survival was observed relative to the controls (non-coated) or the other variously coated embryos. Lower concentrations of Ba or Ca, i.e. 0.0625-0.125%, were avoided because they did not produce a "nice" coating. Ba is known to produce stronger gels with alginate than Ca at the same alginate concentration. In addition, the higher the concentration of the cross-linking agent with the same predetermined alginate concentration, the stronger the gel. As noted earlier, a stronger coating limits the percent of hatched embryos. Another explanation for our findings is that diffusivity decreases with increasing alginate concentration or gel strength. A third, potentially more important explanation is the toxicity of Ba ions to embryos, as reported by Spangenberg and Cherr (1966).

Fig. 3 presents the thickness of the film and jelly coat for coated embryos. Coating thickness was not more than 16% of the embryo's natural Ferret diameter, including the coating (from 0.07 to 0.2 mm), and in general, not thicker than the embryo's natural jelly coats. During the course of natural fertilization, the jelly coat swells when it is immersed in water [14]. In this study, the alginate coating limited the swelling of the jelly coat. After 4 hours of observation, we noted that the thinner the coating, the more swollen the natural jelly coat. The amount of cross-linking agent in the system was much higher than the stoichiometric amount necessary to cross-link the alginate [15,16]. After the spontaneous cross-linking, the strength of the coating film increased and its thickness decreased. After 24 hours, film thickness was reduced by 10 to 40% for the different cross-linking agents used, while the film strengthened. The final outcome of this effect was a limitation of the natural jelly coat's swelling, which was either slowed or prevented by the strengthening of the coating. After 24 hours, the film appears to reach its

maximal strength [16] and the jelly coat stops swelling. The coating prevents the jelly coat from reaching its optimal thickness, as compared to non-coated embryos.

The medium in our case was prone to microbial contamination because the petri dishes were stored open, under non-sterile conditions. It was interesting to note the effect of the alginate coating on the microorganism's development as recorded in relative light units (RLU) vs. time. RLU can easily be transformed to microbial counts with a conversion factor. Using such a conversion we found that about 20 hours after the coating experiments began, total counts were on the order of 101 to 102, reaching values of 2 to 5 x 103 after 48 hours, and average values of 0.7 to 1.5 x 104 after 72 hours. One striking observation was that the non-coated embryos were much more contaminated than their coated counterparts. Normally, microorganisms are glued to the jelly coat, causing considerable contamination of the non-coated embryo [17]. The thin film coating the embryo prevented microorganisms from being glued directly to the jelly coat, thereby reducing contamination. In addition, it is important to note that the alginate-based coating is not a good medium for microorganism development. Moreover, the fact that the coated embryos hatched at a more mature stage than their non-coated counterparts made them more resistant to microbial contamination. Finally, it must be remembered that bacterial growth, which naturally results in oxygen inhibition, causes death, particularly in newly emerged young frogs [17]; in this light, the contribution of the coating becomes much more important.

Using a literature search, we tried to construct a hypothetical model for alginate's reactivity with the natural jelly coat. Light and electron microscopy observation indicated that the alginate coating is glued directly to the exterior of the embryos, i.e. the J3 layer, with no observable gap between the two (Fig. 4).

The coated eggs are immersed at a pH of ~7.4. pKa values for alginic acid may range from 3.4 to 4.4. The pKa for the sialic acids of the jelly coat is ~2.6. Furthermore, the pKa for the glycoprotein amine groups comprising the jelly coat is 7.8 to 7.95. These values leave us with two possible hypothetical alginate interactions: direct interaction between NH3+ on the jelly coat glycoproteins with alginate's COO-, or with calcium as a bridge between acid residues of the alginate and the jelly coat. In addition, hydrogen bonds between the jelly coat and the alginate are a real possibility.

To study the effect of different conditions on the coated embryos' survival, they were introduced into the same medium, which, this time, was sterile. The results of these experiments are shown in Fig. 5. Two main treatment groups appear to emerge: the first reaching asymptotic survival rates of 64 to 70% from 70 hours after fertilization, and the second reaching smaller asymptotic survival values of 34 to 52% at the same time point. This latter group was comprised of coatings cross-linked with 0.5 and 1% BaCl2, again demonstrating barium's toxicity. Since the medium was sterile, the advantages of successful coating were less salient. Although the controls (non-coated) had an initially higher hatching percentage than the coated embryos, the survival prospects of the embryos coated with alginate cross-linked with calcium (0.25, 0.5 or 1%) or barium (0.25%) were better. This can be due to defense against mechanical damage and hatching at a later stage when the embryo is more developed.

To simulate a situation more closely resembles that found in nature, coated embryos immersed into dechlorinated, aerated, circulating tap water. A significant difference between the controls and coated systems was observed. The control exhibited an asymptotic survival percentage of nearly 58, whereas the coated embryos reached no more than 31%. However, in this case the coated embryos held a unique advantage. Survival reached an asymptotic value at least 40 hours after the control. These results can be explained by ICP studies of element content in the different media in which the coated and non-coated embryos were incubated. Due to the concentrations of potent crosslinking agents, the 1/3 CAMMR solution appeared to be most conducive to achieving a weaker alginate-coating gel layer. In other words, since the CAMMR solution contains less calcium, barium, copper, zinc or strontium, reaction with non-crosslinked regions within the gel layer are less likely. A spontaneous crosslinking reaction between alginate and excess calcium salt (as happens here) is known to produce a less-ordered gel relative to a slow crosslinking reaction, which yields a potentially stronger, more ordered network (Nussinovitch et al., 1990). The embryos coated with the stiffer alginate gel coating developed normally within the coating, but exhibited lower hatching rates. This is due to the stronger barrier and hence the more energy the embryo needs to invest in bursting both the jelly coat and the alginate coating via enzymatic and mechanical activity (see previous discussion). Such coating systems, which postpone embryo hatching, can therefore be useful in long-term laboratory experiments. For such uses it is crucial to optimize the working parameters, such as alginate type and concentration, crosslinking agent type and concentration, time of alginate exposure to the crosslinking agent and the composition of the medium in which the embryos

are stored. Other conditions, such as temperature, pH, etc. need to be kept constant and as close as possible to normal biological conditions.

After coating, the excess minerals (observed by ICP) contained within the alginate gel coating are prone to diffusion. Immediately after crosslinking, excess minerals, particularly calcium and sodium, have a tendency to diffuse into the embryo (Table 4), presumably through the ion channels or the membrane itself (Gillespie, 1983; Gillo et al., 1996). Thus, those minerals are expected to increase within the embryo and decrease in the coating membrane. After a while, this increase slows due to the specific activity of the ion channels and the potential ionic diffusion through the gel coat to the surrounding medium (Dascal and Boton, 1990).

The effect of different hydrocolloid coatings on the survival of embryos with time is shown in Fig. 6. The survival percentage is equivalent to the accumulated number of hatching embryos to a maximal or asymptotic survival value, and is the number of embryos left after they begin to die. The accumulated survival percentage of noncoated (control) embryos was ~4.6, 54 h after fertilization, increasing to 66 after 60 h (Fig. 6). Percent survival then decreased to 41 after 78 h and reached an asymptotic value of 30 between 84 and 196 h. Reduced survival percentages could be due to the secretion of nitrates or other substances into the medium by the developing embryos [1]. Moreover, bacteria have been reported to stick to the surface of the outer layer of the JC (J_3) and its removal greatlyeatly rUtheir number [12]. Based on BOD and pH determinations during the experiment, proper aeration conditions and pH prevailed during embryo development, eliminating this as a reason for embryo mortality.

Large differences between the different hydrocolloid coatings were observed. However, all the coatings demonstrated an advantage relative to the noncoated system. The best coating was based on ι-carrageenan gelled with Ca^{2+} reaching an asymptotic survival percentage of ~79, 78 h into the experiment. The κ-Carrageenan coating was second best. No significant difference was observed between the κ-carrageenan and alginate coatings, nor was any detected between the LMP and alginate coatings. All coated embryos appeared to develop normally, similar to noncoated embryos. Moreover, the coating did not prevent the embryo's emergence from its JC but did delay hatching by 18 to 24 h on average. This delay is important for laboratories interested in performing longer-term experiments with embryos. The embryos hatched at a

much more developed stage relative to noncoated embryos (noncoated embryos hatched at stage 33/34, coated embryos at stage 41/42). Thus the formers are less prone to mechanical damage or microbial contamination. In addition, the coating eliminates direct microbial development on the outer surface of the embryo (Kampf et al., 1998) due to the formation of a physical barrier between the J_3 and its surroundings. Thus, coatings could eliminate the need for neomycin sulfate in the media, as suggested by Carroll and Hedrick (1974). In amphibians, the natural JC serves as a heat accumulator, especially at high attitudes where the fertilized eggs are exposed to lower temperatures [18]. Coating the embryo with an artificial gel layer would decrease heat loss by insulating the embryo from its surroundings. Moreover, the artificial gel coating could condense the light rays as they heat the embryo. As stated by Beattie (1980), larger gelatinous capsules around the eggs may increase their chances of survival.

The thickness of the JC 4 and 20 h after coating by the different gums was evaluated by using binocular microscope (Fig. 7). No statistical differences between the same coatings at different times were observed, i.e. after 4 h the thickness of the JC reached its final asymptotic value. The observed thicknesses were 0.16 ± 0.02, 0.22 ± 0.01, 0.19 ± 0.02 and 0.18 ± 0.01 mm for the LMP, ι and κ-carrageenan and alginate coatings resprespectivelyThe thickness of the control was 0.27 ± 0.02. Similar results of natural JC thickness have been reported by Beonnell and Chandler (1996). In other words, the hydrocolloid coating reduces the thickness of the natural JC by eliminating its swelling.

After coating, the hydrocolloid membranes contract, as occurs with many gelling agents after setting, thus preventing the swelling of the natural JC. LMP and alginate coatings undergo a spontaneous cross-linking reaction, and this may be the cause for their profound effect on the JC thickness, while with the carrageenans a slightly slower effect results in a significantly thicker JC. In addition, the hydrocolloid coating solutions contain salts such as Ca, which has been reported to inhibit swelling of the natural JC [18].

The thickness of the coating films and their mechanical properties influenced the presentage of embryo hatch. With ι-carrageenan, the coating is composed of a soft and brittle gel membrane. No tensile test can be performed on such films and the embryo has no problem hatching by "breaking" the coating film, as compared to hatching by breaking the natural JC or the other

coatings (Fig. 8). The second best coating with regards to percent hatch was κ-carrageenan, followed by alginate and LMP. There were no statistical differences between hatching percentages of alginate- and LMP-coated embryos. Differences in the deformability modulus (E_D) of the coated films may play a role in these observations. This property was evaluated by preparing custom-made films with the same chemical composition (see Materials and Methods) and comparing them to those coating the embryos. The E_D, representing gel stiffness, was calculated from the linear portion of the stress-strain curves. The lowest E_D value was found for the κ-carrageenan gel (19.8 ± 4.4 kPa), and there was no significant difference between E_D values of LMP and alginate (33.4 ± 8.2 and 27.0 ± 12.3 kPa, respectively). Similarly, κ-carrageenan gel thickness (Fig. 8) was significantly less than that of LMP or alginate. Thus both E_D and gel thickness values might explain the high hatching percentages observed for κ-carrageenan-coated embryos relative to LMP and alginate. The stress at failure of the different coating films supported these conclusions. The numerical values for strength were 7.5, 6.5 and 76 kPa for κ-carrageenan, LMP and alginate respectively, thus alginate most strongly resists hatching. In addition, the alginate membrane was significantly less brittle than the κ-carrageenan and LMP membranes. In this case, its fracture strain was 0.55, vs. 0.25 and 0.19, respectively. Thus, it can be concluded that embryo hatching depends on the mechanical properties of the coating membranes, the strongest, toughest and least brittle film presenting more resistance to the hatching of the coated embryo. In fact, coating produced a multilayered gel composed of the natural JC layers and the added hydrocolloid layer. At least hypothetically, if the mechanical properties of the JC are important enough to be estimated separately (information which is lacking in textbooks), estimating the gel's coating mechanical properties and combining them with those of the JC multilayered gel should lead to a direct calculation of the stiffness of the JC itself (Ben-Zion and Nussinovitch, 1997).

Regarding the hydrocolloid coatings, it is important to note that no spaces could be detected between the coating and the embryo. In fact, the coatings were glued to the natural JC. Fig. 9a-d demonstrates the thicknesses of the different coatings and their attachment to the embryos. Coating thicknesses were measured by image-processing and the resultant numerical values were 0.05 ± 0.005, 0.03 ± 0.005, 0.017 ± 0.003, 0.15 ± 0.01 mm for LMP, ι and κ-carrageenan and alginate coatings, respectively. These measurements agreed with what was detected under

binocular microscope (see Fig. 8). The shape of the coated embryos using the different hydrocolloid coatings is demonstrated in Fig. 10. While LMP and alginate contributed to the smoothness of the external coatings, the carrageenans created many folds on the surface. Whether this depends on coating thickness or results from a slower gelation is not yet clear.

References

1. M. Wu and J. Gerhart, Methods Cell Biol. 36 (1991) 3.

2. J.L. Hedrick and T.J. Nishihara, Electron Microscopy Technique. 17 (1991) 319.

3. P.D. Nieuwkoop and J. Farber, In Normal table of Xenopus laevis (Daudin). Pp. 162 New York & London: Garland Publishing, Inc. 1994.

4. G. Waes, Milchwissenschaft, 12(39) (1984) 707.

5. A. Nussinovitch and N. Kampf, Lebensmittel-Wissenchaft und-Technologie, 26 (1993) 469.

6. A. Nussinovitch and V. Hershko, Carbohydrate Polymers, 30 (1996) 185.

7. V. Hershko, D. Weisman and A. Nussinovitch, J. of Food Science, 63 (2) (1998) 317.

8. V. Hershko and A. Nussinovitch, J. Agricultural and Food Chemistry, 46 (8) (1998) 2988.

9. N. Kampf and A. Nussinovitch, Polymer Networks, June 28 - July 3, Trondheim, Norway. (1998).

10. F. Lim and A.M. Sun, Science. 210 (1980) 908.

11. R.J. Phillips, J. The Institute of animal Technology, 30(1) (1979) 11.

12. E.J. Carroll (Jr.) And J.L. Hedrick, Developmental biology, 38 (1974) 1.

13. J.V. Spangenberg and G.N. Cherr, Environmental Toxicology and chemistry, 15(10)(1966) 1769.

14. R.S. Seymour, Israel J. of Zoology. 40 (1994) 493.

15. M. Glicksman, In: Gum Technology in the Food and Other Industries, pp. 152, Chapman and Hall, London UK, 1969.

16. A. Nussinovitch, In: Gum Technology in the Food and Other Industries, pp. 176, Chapman and Hall, London, UK, 1997.

17. J.S. Davys, Animal Technology, 37(3)(1986) 217.

18. Beattie, R.C. (1980) A physico-chemical investigation of the jelly capsules surrounding eggs of the common frog (*Rana temporaria temporaria*). J. Zool. Lond., 190,1-25.

19. Banerjee, M., Chakrabarty, A. and Majumdor, S.K. (1982) Immobilization of yeast cells containing -galactosidase. Biotech. Bioeng., 24,1839-1850.

20. Brodelius, P. and Nilsson, K. (1980) Entrapment of plant cells in different matrices. Fed. Euro. Biochem. Soc. Lett., 122, 312-6.

21. Bucke, C. (1983) Immobilized cells. Philosophical Transactions of the Royal Society, London, Series B, 300, 369-89.

22. Cheetham, P.S.J. (1980) Developments in the immobilization of microbial cells and their applications. In: Topics in Enzyme and Fermentation Biotechnology, vol. 4 (Wiseman, A. ed.), Chichester Ellis Horwood Ltd, pp. 189-238.

23. Chibata, I. (1981) Immobilized microbial cells with polyacrylamide gel and carrageenan and their industrial applications. Am. Chem. Soc. Symp. Ser., 106, 187-202.

24. Dacunzo, A., Dealteriis, E., and Maurano, F. (1996) D-amino-acid oxidase from *Trigonopsis variabilis* immobilization of whole cells in natural polymeric gels for glutaryl-7-aminocephalosporanic acid production. J. Ferment.Bioeng., 81(2), 138-42.

25. Dainty, A.L., Goulding, K.H., Robinson, P.K., Simpkins, I. and Trevan, M.D. (1986) Stability of alginate-immobilized algal cells. Biotechnol Bioeng., 28, 210-216.

26. Dascal, N., and Boton, R. (1990) Interaction between injected Ca^{2+} and intracellular Ca^{2+} stores in *Xenopus* oocytes. FEBS, 267(1), 22-24.

27. Deriso, L., Dealteriis, E., and Lacara, F. (1996) Immobilization of *Bacillus Acidocaldarius* whole-cell Rhodanese in polysaccharide and insolubilized gelatin gels. Biotec. Appl. Biochem., 23, 127-31.

28. Dobreva, E., Ivanova, V., and Tonkova, A. (1996) Influence of the immobilization conditions on the efficiency of -amylase production by *Bacilus Licheniformis*. Process biochem., 31(3), 229-234.

29. Douglas, A., Dawson, T., Wayne, S. and Edward, C.S. (1992) Laboratory care and breeding of the African clawed frog. Lab. Anim., 21(4),31-36.

30. Hannoun, B.J.M. and Stephanopoulos, G. (1986) Diffusion coefficient of glucose and ethanol in cell-free and cell-occupied calcium alginate membranes. Biotechnol. Bioeng., 28,829-835.

31. Hatanaka, T., Yasuda, T., Yamaguchi, T. and Sakai, A. (1994) Direct growth of encapsulated somatic embryos of coffee (*Coffea canephora*) after cooling in liquid nitrogen. Croi-lett., 15,47-52.

32. Henriques, U. (1964) Breeding of *Xenopus laevis* Daudin. Acta Endocrin., 90,89-98.

33. Gillespie, J.I. (1983) The distribution of small ions during the early development of *Xenopus laevis* and *Ambystoma mexicanum* embryos. J. Physiol., 344, 359-377.

34. Gillo, B., Sealfon, S.C. and Minke, B. (1996) Pharmacology of capacitative Ca^{2+} entry in *Xenopus* oocytes. J. Photochem. Photobiol., 35, 77-82.

35. Green, K.D., Gill, I.S., and Khan, J.A. (1996) Microencapsulation of yeast cells and their use as a biocatalyst in organic solvents. Biotec. and Bioeng., 49(5), 535-43.

36. Guardiola, J., Iborra, J.L., and Rodenas, L. (1996) Biotransformation from Geraniol to Nerol by immobilized Grapevine cells (*V-Vinifera*). Appl. Biochem.Biotec., 56(2), 169-80.

37. Jen, A.C.,Wake, M.C. and Mikos, A.G. (1996) Review--hydrogels for cell immobilization. Biotec. Bioeng., 50(4), 357-64.

38. Kamboj, R.C., Raghav, N., and Nandal, A. (1996) Properties of cathepsin-B immobilized in calcium alginate beads. J. Chem. Technol. Biotechnol., 65 (2), 149-55.

39. Khachatourians, G.G., Brosseau, J.D. and Child, J.J. (1982) Thymidine phosphorylase activity of anucleate minicells of *E. coli* immobilized in an agarose gel matrix. Biotechnol. Lett., 4,735-740.

40. Kim, M.N., Ergan, F. and Dhulster, P. (1982) Steroid modification with immobilized mycelium of *Aspergillus phoenics*. Biotechnol. Lett., 4,233-238.

41. Kluge, M., Klein, J. and Wagner, F. (1982) Production of 6-aminopenicillanic acid by immobilized *Pleurotus ostreatus*. Biotechnol. Lett., 4,293-296.

42. Krisch, J. and Szajani, B. (1996) Aceacid fermentation of *Acetobacter-Aceti* as a function of temperature and pH. Biotechnol. Lett., **18**(4),393-6.

43. Kojima, T., Hashimoto, K., Ito, S., Hori, T., Tomizuka, T. and Oguri, N. (1990) Protection of rabbit embryos against fracture damage from freezing and thawing by encapsulation in calcium alginate gel. J. Exp. Zool., 254(2),186-191.

44. Krouwel, P.G., Harder, A. and Kossen, N.W.F. (1982) Tensile stress-strain measurements of materials used for immobilization. Biotechnol. Lett., 4,103-108.

45. Mattiasson, B. (1983) Immobilized Cells and Organelles, vols. 1 & 2. CRC Press, Boca Raton, FL.

46. McNeely, W.H. and Pettitt, D.J. (1973) Algin In: Industrial gums. Whistler, R.L. and (BeMiller, J.N. ed.). Academic Press, NY.

47. Nussinovian, I. J. and Mizrahi, S. (1997) Effect of hydrocolloids content and minerals on gels mechanical properties. Food Hydrocolloids, 4, 257-65.

48. Nussinovitch, A. (1994) Resemblance of immobilized *Trichoderma viride* fungal spores in an alginate matrix to a composite material. Biotech Prog, 10,551-554.

49. Nussinovitch, A., Nussinovitch, M., Shapira, R. (1994) Influence of immobilization of bacteria, yeasts and fungal spores on the mechanical properties of agar and alginate gels. Food Hydrocolloids, 8,361-72.

50. Nussinovitch, A. (1997) Immobilization and encapsulation. In: Hydrocolloid applications. Chapman and Hall, London. Pp. 247-264.

51. Redenbaugh, K., Paasch, B.D., Nichol, J.W., Kossler, M.E., Viss, P.R. and Walker, K.A. (1986) Somatic seeds: encapsulation of asexsual plant embryos. Biotechnol. 4, 797-801.

52. Ruddock, P.A. and Ruffle, W.G. (1972) The care and maintenance of a breeding *Xenopus* colony. J. Instit. Anim. Tech., 23(2), 83-90.

53. Spangenberg, J.V. and Cherr, G.N. (1966) Developmental effects of barium exposure in a marine bivalv (*Mytilus californianus*). Env. Toxic. Chem., 15(10), 1769-77.

54. Stocklein, W., Eisgruber, A. and Schmidt, H.L. (1983) Conversion of L-phenylalanine to L-tyrosine by immobilized bacteria. Biotechnol. Lett., 5, 703-708.

55. Tampion, J. and Tampion, M.D. (1987) Immobilized Cells: Principles and Applications, Cambridge University Press, Cambridge.

56. Vorlop, K.D. and Klein, J. (1981) Formation of spherical chitosan biocatalysts by iontropic gelation. Biotechnol. Lett., 3, 9-14.

57. Walsh, P.K., Isdell, F.V. and Noone, S.M. (1996) Microcolonies in alginate and carrageenan gel particles effect of physical and chemical properties of gels. Enz.Microb. Technol., 18(5), 366-372.

58. Wang, H.Y. and Hettwer, D.J. (1982) Cell immobilization in -carrageenan with tricalcium phosphate. Biotechnol. Bioeng., 24, 1827-1838.

59. Wiksstrom, P., Szwajcer, E., and Brodelius, P. (1982) Formation of -keto acids from amino acids using immobilized bacteria and algae. Biotech. Lett., 4, 153-8.

60. Baldwin, E.A. (1994) Edible coating for fresh fruit and vegetable: past, present and future. In: Edible coating and films to improve food quality. (J. Krochta, E. Baldwin and M. Nisperos-Carriedo, eds). Technomic, Basel, Swizerland.

61. Ben-Zion, O. and Nussinovitch, A. (1997). A prediction of the compressive deformabilities of multi-layered gels and texturized fruit, glued together by three difee different achniques. Food Hydrocolloids, 11 (3), 253-260.

62. Beonnell, B.S. and Chandler, D.E. (1996) Egg jelly layers of *Xenopus laevis* are unique in ultrastructure and sugar distribution. Mol. Rep. Dev. **44**, 212-220.

63. De Laat, S.W., Wouters, W., Da Silva Pimenta Guarda, M.M. and Da Silva Guarda, M.A. (1975) Intracellular ionic compartmentation, electrical membrane properties, and cell membrane permeability before and during first cleavage in the *Ambystoma* egg. Exp. Cell Res. **91**, 15-30.

64. Forini, C., Tel-Or, E., Bar, E. and Grilli-Caiola, M. (1991) Effect of antibiotic treatment on *Azolla-Anabaena* and *Arthrobacter*. Plant Soil **137**,151-155.

65. Gotteschalk, A. (1966) Glycoproteins. Elsevier, Amsterdam, pp. 63-177.

66. Hedrick, J.L. and Hardy, D.M. (1991) Isolation of extracellular matrix structures from Xenopus laevis oocytes, eggs and embryos. Meth. Cell. Biol. **36**, 231-247.

67. Hershko, V. and Nussinovitch, A. (1998 b). Relationships between hydrocolloid coating and mushroom structure J. Agric. Food Chem. **46**(8), 2988-2997.

68. IFT (1991) New from Mitsubishi Annu. Meet. Food Expl. Prog. Exhibit Directory, Dallas Convention Center, June 1-5, Chicago, IL.

69. Kampf, N. and Nussinovitch, A. (1998). Gum coating of cheeses. Osaka City University International Symposium. Joint Meeting with the 4th International Conference of Hydrocolloids. October 4 - October 10, Osaka, Japan.

70. Kampf, N., Zohar, C. and Nussinovitch, A. (1998). Alginate coating of *X. laevis* embryos. Osaka City University International Symposium. Joint Meeting with the 4th International Conference of Hydrocolloids. October 4 - October 10, ober 10, Osaka, Japa.U71. Kampf, N., Zohar, C. a

Biotech. Progr. (submitted).

72. Nussinovitch A. and Kampf N. 1993. Shelf-Life extension and conserved texture of alginate-coated mushrooms (Agaricus bisporus). Lebensm. -Wiss. u.-Technol. **26**, 469-475.

73. Nussinovitch, A., Nussinovitch, M. and Shapira, R. (1994) Influence of immobilization of bacteria, yeasts and fungal spores on the mechanical properties of agar and alginate gels. Food Hydrocolloids **8**, 361-372.

74. Phillips, R.J. (1979) The care and induced breeding of *Xenopus laevis*. J. Inst. Anim. Technol. **30**(1), 11-16.

75. Shimoda, Y., Kitajima, K., Inoue, S. and Inoue, Y. (1994) Calcium ion binding of three different types of oligo/polysialic acids as studied by equilibrium dialysis and circular dichroic methods. Biochemistry **33**, 1202-1208.

76. Swenson, H.A., Miers, J.C. and Schultz, T.H. (1953) Pectinate and pectate coatings. Application to nut and fruit products. Food Technol. **7**, 232-235.

76. Torres, J.A., Hall, E.G. and Karel, M. (1985) Microbial stabilization of intermediate moisture food surfaces. Control of surface preservative concentration. J. Food Proc. Pres., **9**, 75.

77. Yurewicz, E., Oliphant, G. and Hedrick, J. (1975) The macromolecular composition of *Xenopus laevis* eggs. Biochemistry **14**(14), 3101-3107.

Claims

1. A method of coating a cell characterised in that the cell is placed in a solution of hydrocolloid and, after removing the cell from the hydrocolloid solution, is placed in a cross-linking solution, to thereby provide the cell with a thin coating of the hydrocolloid.
2. A method as defined in Claim 1, wherein the hydrocolloid is an alginate.
3. A method as defined in Claim 1, wherein the alginate is Na-alginate.
4. A method as defined in Claim 1, wherein the hydrocolloid is LMP.
5. A method as defined in Claim 1, wherein the hydrocolloid is selected among κ-carrageenan or ι-carrageenan.
6. A method as defined in any of Claims 1 to 5, characterised in that the hydrocolloid solution is in CAMMR.
7. A method as defined in any of Claims 1 to 6, wherein the cell is a Xenopus laevis egg and embryos.
8. A method as defined in any of Claims 1 to 7, wherein the cross-linking solution is a solution of Ca, Ba or K ions.
9. A method as defined in Claim 8, wherein the cross-linking solution is a solution of $CaCl_2$ or $BaCl_2$ or KCl.
10. A method as defined in Claim 9, wherein the cross-linking solution of $CaCl_2$ or $BaCl_2$ is at a concentration of from 0.25 to 1 wt. % and KCl solution is at a concentration of 0.5 %.
11. A method as defined in any of Claims 1 to 10, wherein said thin layer is up about 50 μm in thickness.
12. A method of postponing hatching of Xenopus laevis embryos comprising applying a thin coating of an hydrocolloid to a Xenopus laevis egg and cross-linking said hydrocolloid.
13. A method as defined in any of Claims 1 to 3 and 6 to 12 wherein the alginate has a high M content.
14. A method as defined in Claim 13 wherein the M content of the alginate is from about 29 to about 61 %.
15. A cell having a thin coating of a hydrocolloid according to any of Claims 1 to 14.

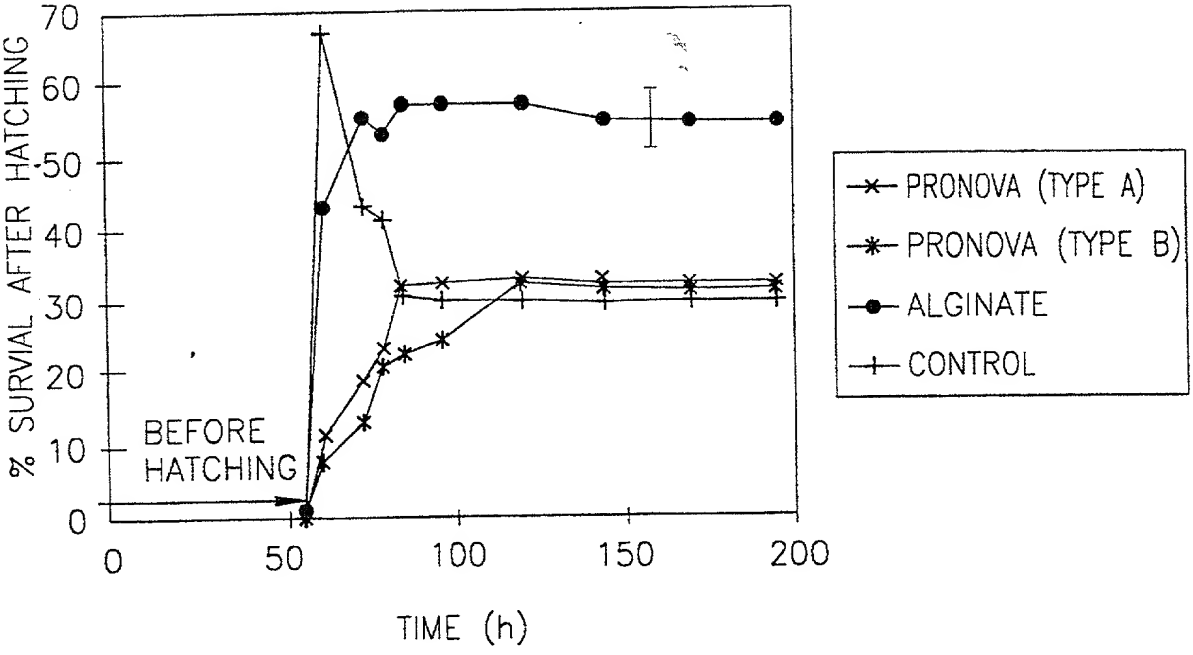

FIG.1

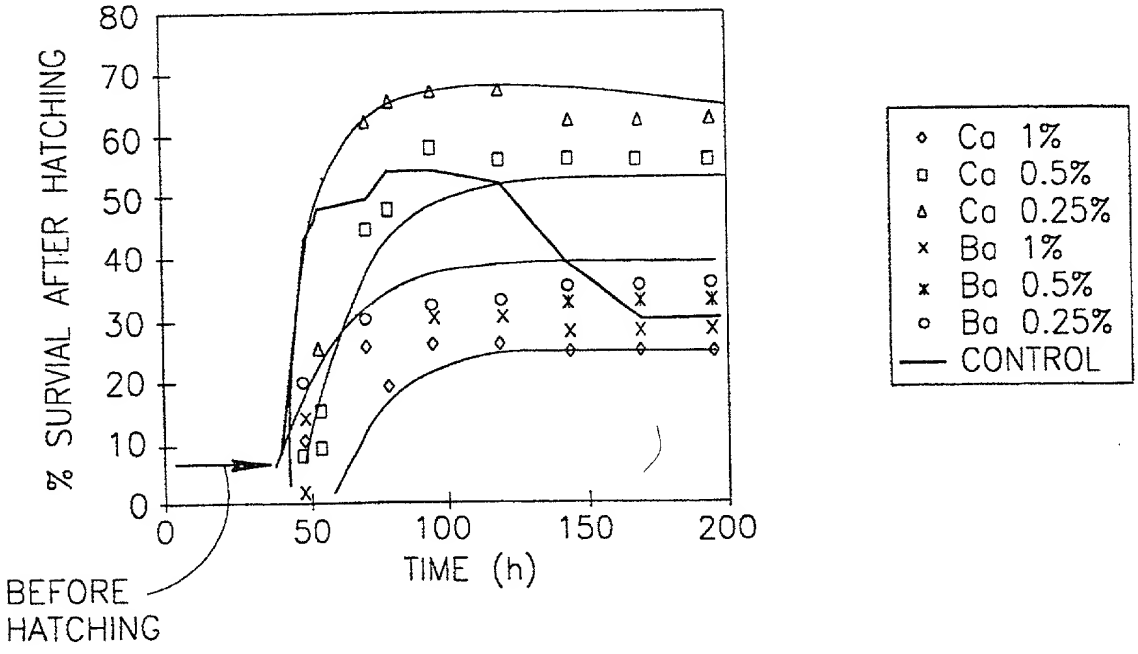

FIG.2

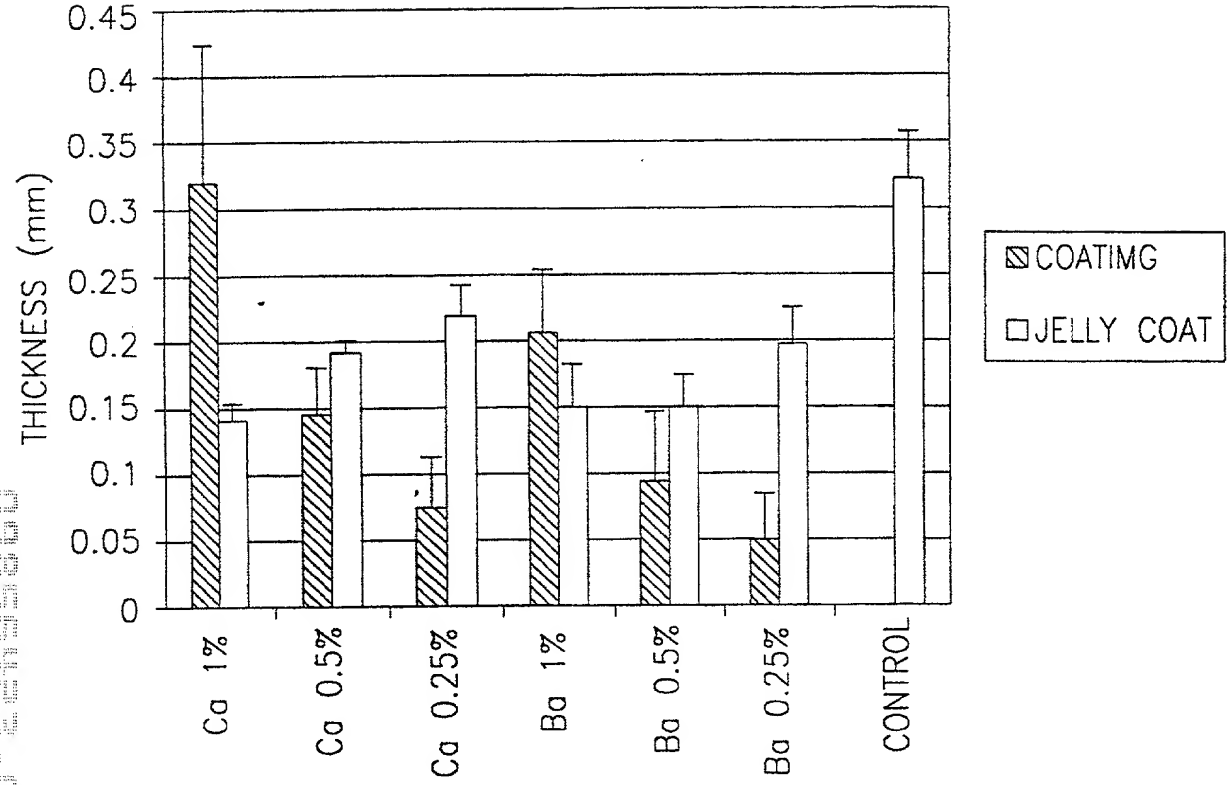

FIG.3

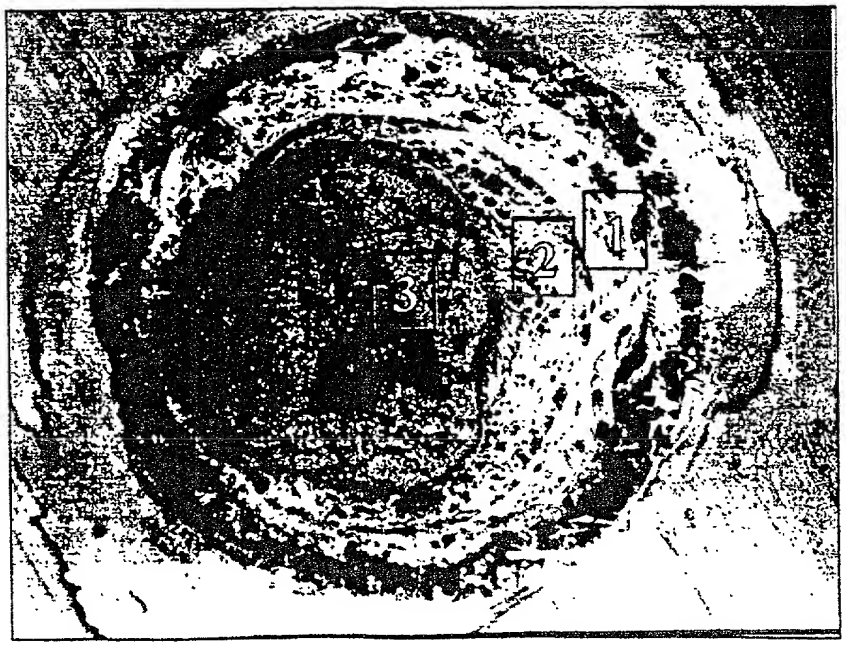

FIG.4

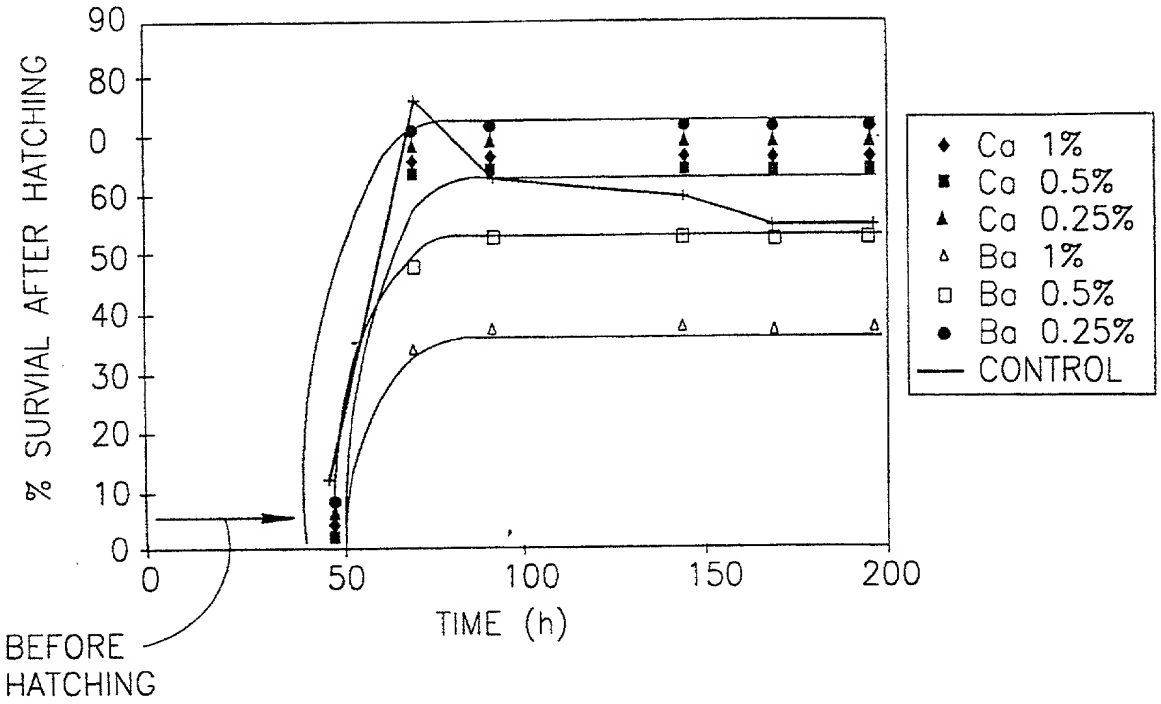

FIG.5

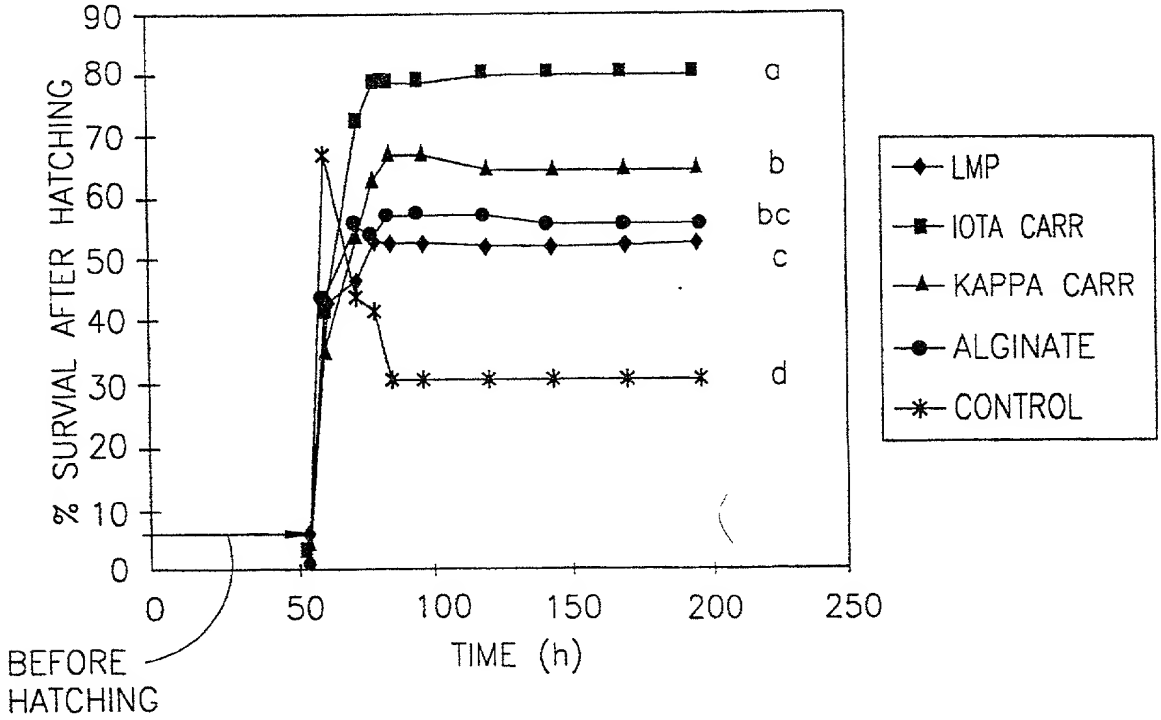

FIG.6

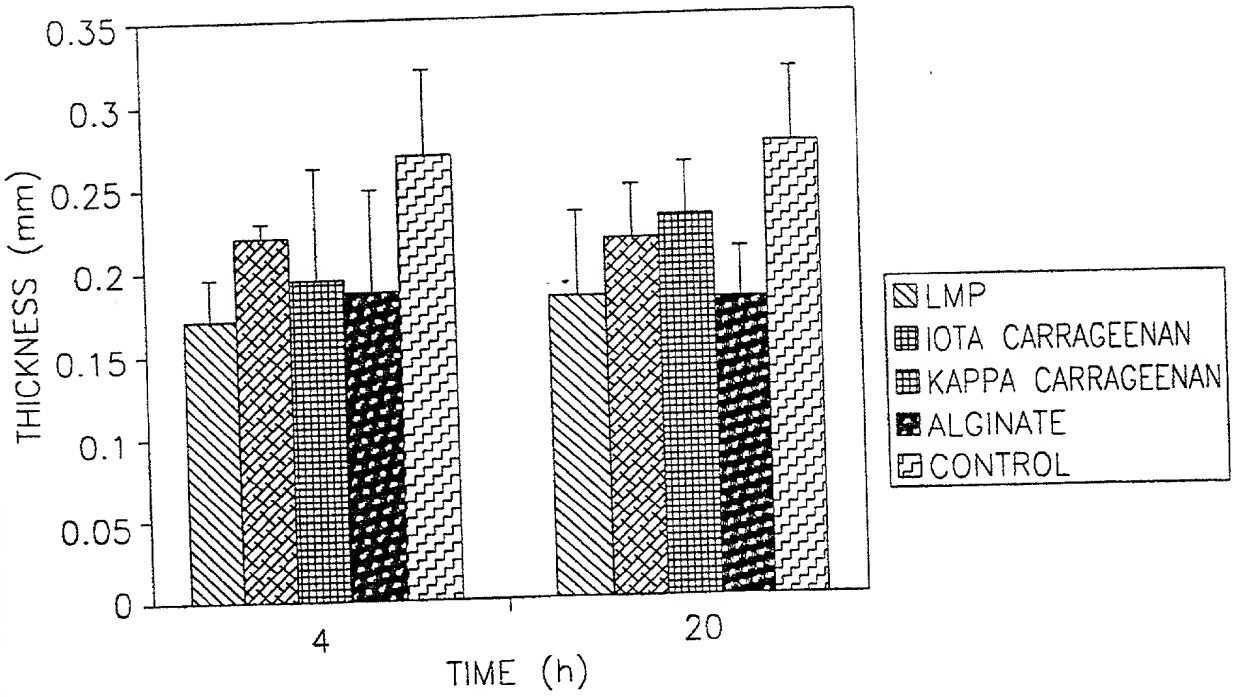
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
THICKNESS (mm)
4
20
TIME (h)
LMP
IOTA CARRAGEENAN
KAPPA CARRAGEENAN
ALGINATE
CONTROL

FIG.7

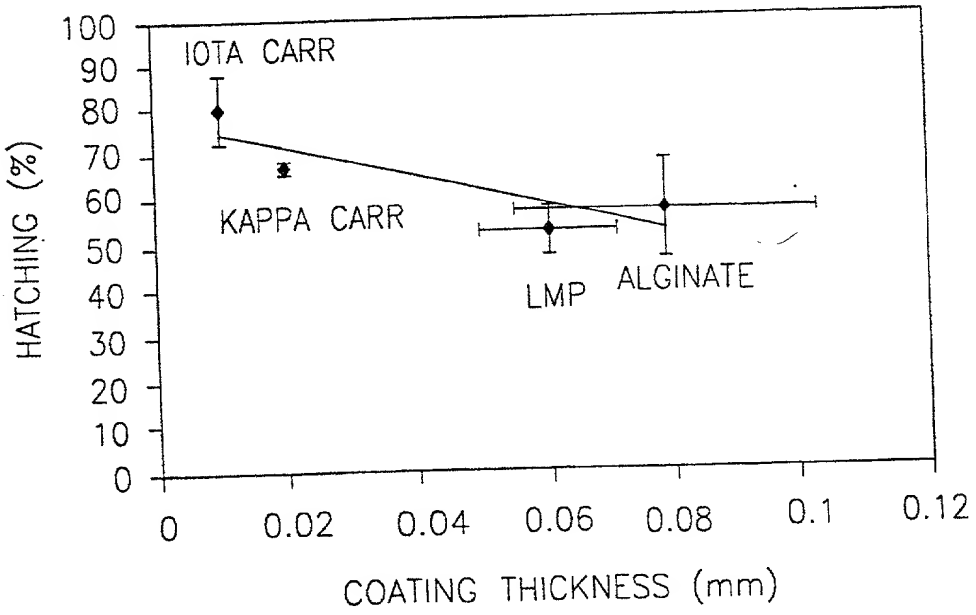
100
90
80
70
60
50
40
30
20
10
0
HATCHING (%)
IOTA CARR
KAPPA CARR
LMP
ALGINATE
0
0.02
0.04
0.06
0.08
0.1
0.12
COATING THICKNESS (mm)

FIG.8

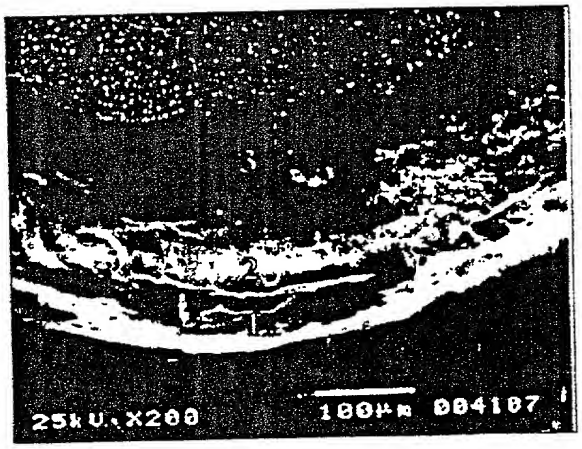

FIG.9a

FIG.9b

FIG.9c

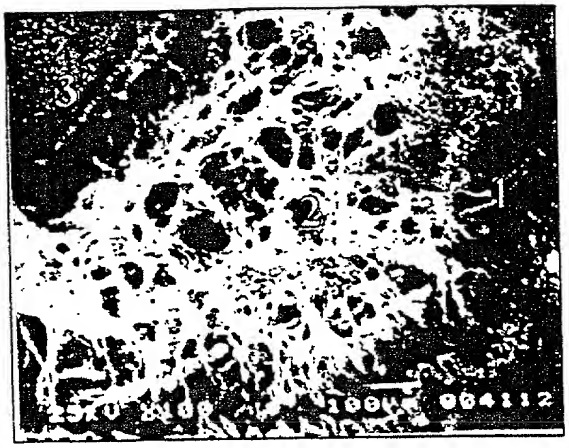

FIG.9d

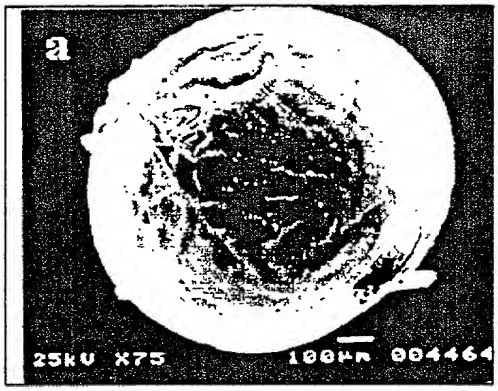

FIG.10a

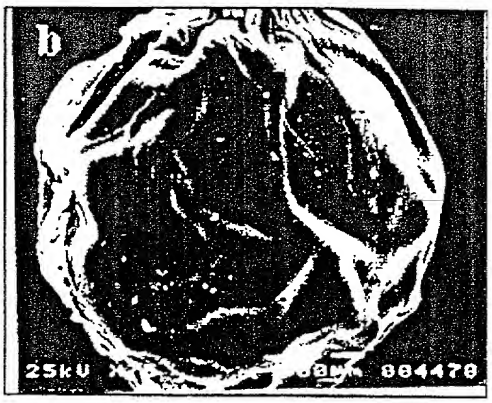

FIG.10b

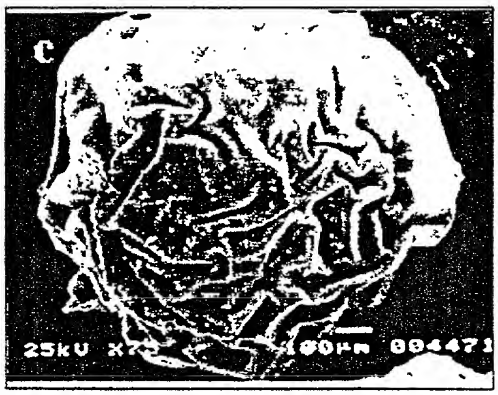

FIG.10c

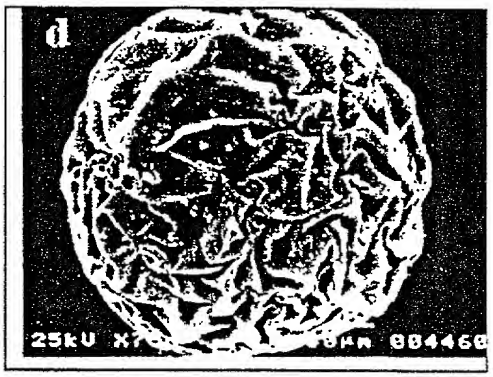

FIG.10d

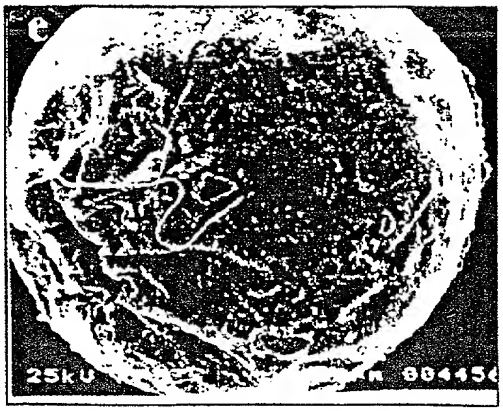

FIG.10e

919.1002

DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

[ ] Declaration submitted with initial filing
[X] Declaration submitted after initial filing (surcharge (37 CFR 1.6(c) required))

First Named Inventor: Anna NUSSINOVITCH

COMPLETE IF KNOWN:

Application Number: 09/856,423

Filing Date: May 21, 2001

Group Art Unit:

Examiner Name:

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

HYDROCOLLOID COATING OF CELLS
(Title of the Invention)

the specification of which
[ ] is attached hereto
OR
[X] was filed on (MM/DD/YY) 05/21/01 as United States Application Number or PCT International Application Number 09/856,423 and was amended on (MM/DD/YY) 05/21/0 (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above. I acknowledge the duty to disclose information which is material to patentability of this application as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YY)	Priority Not Claimed	Certified Copy Attached? Yes	No

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YY)
60/104,118	October 13, 1998 (10/13/98)

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YY)	Parent Patent Number (if applicable)
PCT/IL99/00541	October 13, 1999 (10/13/99)	

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

[X] Customer Number 21831

Direct all correspondence to:

[X] Customer Number 21831

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Nir KAMPF

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